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Downregulation of the ERK 1 and 2 mitogen activated protein kinases using antisense oligonucleotides inhibits proliferation of porcine vascular smooth muscle cells

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Abstract

The current model of the arterial response to injury suggests that proliferation of vascular smooth muscle cells is a central event. Mitogen activated protein kinases are part of the final common pathway of intracellular signalling involved in cell division and thus constitute an attractive target in attempting to inhibit this proliferation. We hypothesised that antisense oligonucleotides to mitogen activated protein kinase would inhibit serum induced smooth muscle cell proliferation by downregulating the protein. Porcine vascular smooth muscle cells were cultured and an antisense oligonucleotide sequence against the ERK family of mitogen activated protein kinases (AMK1) was introduced by liposomal transfection. Sense oligonucleotides and a random sequence were used as controls. Proliferation was inhibited by AMK1 versus the sense controls, as assessed by tritiated thymidine incorporation ($P < 0.01$). Immunoblots revealed downregulation of the target protein by AMK1 by 63% versus the sense control ($P < 0.05$). In conclusion, antisense oligonucleotides specifically inhibited proliferation and downregulated the target protein. This is consistent with a central role for mitogen activated protein kinases in vascular smooth muscle cell proliferation in the porcine model. In addition, the data suggest a possible role for antisense oligonucleotides in the modulation of the arterial injury response.

Keywords: Protein kinases; Second messengers; Signal transduction; Smooth muscle; Vasculature; Cell proliferation; Antisense oligonucleotides

1. Introduction

The formation of an arterial neointima is a characteristic feature of the vascular response to diverse forms of injury [1]. It is implicated in such disparate processes as atherosclerosis [2], the restenotic response following percutaneous transluminal coronary angioplasty [1] and vein graft failure following coronary artery bypass grafting [3]. While many cell types are involved in the process of neointima formation, the vascular smooth muscle cell (VSMC) is the most prominent participant [2,4]. In response to early events occurring as a result of arterial injury, basic fibroblast growth factor (bFGF) is released which causes medial VSMCs both to migrate towards the lumen and to divide [5]. Later events include an increased sensitivity of these migrated VSMCs to a variety of other stimulatory cytokines, such as epidermal growth factor and platelet derived growth factor (PDGF), released from inflammatory cells, platelets and probably from the VSMCs themselves [2]. PDGF may be particularly relevant since it has been shown to be both a mitogen and to promote VSMC migration, a central process in neointimal formation [6,7]. Several attempts to limit neointimal formation have employed substances known to modulate these cytokines or their receptors [8,9]. However, while

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a number of these have been successful in small animal models, clinical tests have proved generally disappointing [10–12].

An alternative approach to preventing the proliferation and migration of VSMCs may be to influence the downstream intracellular signalling events responsible for transducing the signals from the various growth factor receptors. Possible candidates for such manipulation are the mitogen activated protein kinases (MAPK). These serine-threonine kinases are a group of highly conserved and ubiquitously expressed proteins that have been shown to become activated by phosphorylation in response to numerous different stimuli [13,14]. There are at least five subfamilies of mammalian MAPK: the p38 family, the p42 and p44 group, also known as ERK1 and ERK2, MAPKjnk, MAPKerk3/4 and MAPKerk5, and of these ERK1 and 2 are the most extensively studied. The best-characterised mode of activation is by binding of a ligand to a receptor with subsequent autophosphorylation. This event promotes the conversion of membrane-associated Ras-GDP to the activated GTP form [15]. A kinase cascade is then initiated with phosphorylation and activation of Raf-1, MEK (MAPK/ERK kinase) and ultimately ERK1 and ERK2 themselves [14]. Once activated, they then translocate to the nucleus where they initiate the transcription of several immediate early genes involved in cellular proliferation and growth, such as *c-fos*, *c-jun* and *c-myc* [16]. While it is known that these MAPK are instrumental in transducing tyrosine kinase receptor growth signals in VSMCs, for instance from the epidermal growth factor receptor [17], it also appears that they may serve to integrate input from a variety of different receptor types [13,18]. We hypothesised that manipulation of ERK activity could be a particularly effective tool in modulating VSMC growth responses, since many different receptor systems feed into ERK as the final common pathway. We therefore studied the effect of downregulation of ERK, using antisense oligodeoxynucleotides (ODNs), on the proliferation of serum stimulated vascular smooth muscle cells in vitro.

2. Materials and methods.

Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), antibiotics and trypsin-EDTA solution were all purchased from GIBCO. Lipofectin reagent was from Life Technologies Ltd. ODNs were synthesised by the King's College School of Medicine and Dentistry oligonucleotide synthesis service using a Beckman Instruments Oligo 1000 M DNA synthesiser. Lactate dehydrogenase (LDH) assay was performed using a kit from Sigma Chemical Company. 6-^{3}H thymidine for the cellular proliferation assay was from Amersham International and diaminobenzoic acid (DABA) used in the DNA quantitation was from Sigma Chemical Company. Reagents for the assay of protein concentration by the Bradford method, were purchased as a kit from Bio-Rad, as was bisacrylamide for the production of polyacrylamide gels used in the immunoblotting experiments. The primary antibody used for the detection of p42 and p44 MAPK was a mouse anti-human monoclonal from Zymed (clone Z033) and the rabbit anti-mouse horseradish peroxidase linked secondary antibody was from Amersham, as was the enhanced chemiluminescence (ECL) reagent. Polyvinylidene fluoride (PVDF) membrane, used in the Western blotting experiments, was from BioRad. All other general laboratory reagents were from Sigma Chemical Company.

2.1. Vascular smooth muscle cell culture

Porcine VSMC cultures were prepared as previously described [19]. Briefly, pig aortae were opened, stripped of endothelium using a rubber policeman and the medial smooth muscle layer carefully peeled away from the adventitia. Explants, 1 mm^2, of the smooth muscle layer were then seeded into T75 flasks and cultured in DMEM containing 10 mmol/l L-glutamine, 100 U/ml of penicillin and streptomycin and 15% FCS. All cultures were performed at 37°C in a 95% O_2/5% CO_2 atmosphere. Once the VSMCs had grown out from the explants and the flask was more than 50% confluent, cells were trypsinised and passaged between two and four times. The identity of the cells was confirmed by the characteristic 'hills and valleys' appearance and also by immunostaining for smooth muscle α-actin as previously described [20]. This confirmed the cultures to be greater than 95% smooth muscle cells (data not shown). At the final passage before use, cells were transferred to 12 well plates for the tritiated thymidine uptake experiments, chamber slides for the fluorescence microscopy, or T25 plates for the immunoblotting experiments. All transfection experiments were performed at approximately 70% confluence.

2.2. Oligodeoxynucleotides

Porcine p42 and p44 MAPK have not been cloned and sequenced, however ERK is highly conserved between species and a search of the GenBank database using the GCG package revealed that the region of the translation initiation site of the gene is identical in rat and human. ODN sequences were therefore based on these identical sequences. The antisense ODN used was an 18-mer directed against a sequence starting at the initiation codon (**AMK1** 5′–GCC GCC GCC GCC GCC AT–3′). Sense ODN (**SMK1** 5′–ATG GCG GCG GCG GCG GC–3′) and a random ODN (**RAN5**′–CGC GCG CTC GCG CAC CC–3′) were

also utilised as controls. All bases were protected by phosphorothioation and one batch of AMK1 was tagged with fluorescein, for use in fluorescence microscopy experiments. The antisense sequence used has been characterised in detail and demonstrated to selectively downregulate the p42 and p44 isoforms of MAPK, without affecting upstream or downstream elements in the transduction cascade [21].

2.3. Liposomal transfection

Prior to transfection, cultured VSMCs were quiesced in serum-free medium for 72 h. All transfections were carried out under sterile conditions. ODNs were mixed with antibiotic and serum-free medium to a concentration of 0.8 μM. This was then mixed with an equal volume of medium containing 80 μg/ml of lipofectin, vortexed and allowed to stand for 15 min at room temperature. Two hundred microlitres of this mixture was then added to each well of a 12 well plate, 100 μl to chamber slides for the fluorescence microscopy experiments and 1.25 ml to the T25 plates. An equal volume of medium was then added giving a final concentration of 0.2 μM for the ODNs and 20 μg/ml of lipofectin. The concentration of ODN chosen was based on previously published work [20] and also preliminary experiments which revealed that a higher concentration of 0.4 μM showed evidence of non-specific toxicity (data not shown). The cells were incubated in ODN/lipofectin transfection medium for a further 6 h with gentle agitation every 2 h. After 6 h the transfection medium was removed and the cells washed in phosphate buffered saline (PBS). This was replaced with twice the transfection volume of medium, now containing 15% FCS and penicillin and streptomycin, with 0.2 μM of the appropriate ODN added but without lipofectin. Cells were cultured for a further 48 h at 37°C/5% CO2 prior to harvesting. For the tritiated thymidine incorporation experiments, 1 μCi/ml of ^{3}H-thymidine was also added to the medium 18 h prior to the termination of the cultures.

2.4. Fluorescence microscopy

VSMCs were plated onto chamber slides and transfected using either the fluorescein linked AMK1 or the non-fluorescein tagged oligonucleotide as a negative control. After 24 and 48 h of incubation, the slides were viewed by epi-illumination on a Zeiss Axioskop fluorescence microscope and photographed using Kodak ektachrome 160T film.

2.5. Determination of cell viability

The effect of the different ODNs on cell viability was studied to ensure that any differences in growth between the groups was not due to cellular toxicity. This was assayed by quantification of LDH release into the culture supernatant using a commercial kit, as per the manufacturers instructions. All LDH determinations were performed in triplicate. In addition, all cultures were examined by phase contrast microscopy prior to harvesting, looking for any evidence of abnormal morphology or detachment of cells.

2.6. Assessment of cell proliferation

Cell proliferation was assayed using the tritiated thymidine incorporation assay [22]. Following transfection and addition of 1 μCi/ml of ^{3}H-thymidine, as described above, cells were harvested using ice cold 10% trichloracetic acid. Following centrifugation and ethanol extraction of the samples, duplicate 50 μl aliquots were counted using a Beckman Instruments scintillation counter. DNA content of the samples was determined using the DABA fluorescence method of Kissane and Robins [23]. Results were expressed as disintegrations per min per μg of DNA and experiments were repeated at least in triplicate.

2.7. Western blotting

For the assessment of ERK protein levels, VSMCs were cultured in T25 plates as described above. At termination, cells were washed twice with ice cold PBS and then 1 ml of extraction buffer was added to the plates (20 mmol/l glycerophosphate, 20 mmol/l NaF, 2 mmol/l EDTA, 0.2 mmol/l sodium vanadate, 10 mmol/l benzamidine, 25 μg/ml leupeptin, 50 μg/ml phenylmethylsulphonyl fluoride, 0.3% by vol. β-mercaptoethanol, pH 7.5). Plates were scraped and the extracted samples concentrated using Centricon 30 spin columns (Amicon) as per the manufacturer's instructions. Protein content was determined using the Bradford protein assay kit. Following addition of 3 × Laemmli buffer (0.33 mol/l Tris/HCL, 10% sodium dodecylsulphate, 13% glycerol, 0.1 mol/l dithiothreitol, 0.13 mg/ml bromophenol blue), samples were denatured by boiling for 5 min and then resolved on 12% polyacrylamide gels and transferred to PVDF membranes. Equal quantities of protein were loaded in each lane, determined by the Bradford assay and this was further checked by Ponceau staining of the PVDF membranes. Any membranes in which there appeared to be unequal amounts of protein in the lanes were discarded. Non-specific binding to the membranes was blocked using 5% milk powder in PBS/0.05% Tween 20 (PBS/Tween) for 1 h and then anti-MAPK antibody was added to a concentration of 1/3000 and the membranes exposed overnight at 4°C. Following three washes in PBS/Tween the membranes were exposed to the secondary antibody, diluted 1 in 5000 in PBS/

Tween/1% milk powder for 1 h at room temperature. Following three further washes in PBS/Tween, the membranes were then developed using ECL and recorded on Kodak Ektachrome film. Quantitation of the results was by scanning using a Bio-Rad GS3000 densitometer connected to an Apple Power Macintosh computer, followed by analysis using the Bio-Rad Molecular Analyst software. Initial studies had shown that this system produced a linear relationship between quantity of ERK protein run on the gel and the intensity of the band ($r^2 = 0.98$, data not shown)

2.8. Statistical methods

Data are presented as means ± SEM. Statistical comparisons between pairs of groups was performed using the unpaired *t* test. Comparisons of multiple groups was done using one way ANOVA, with subsequent testing for individual intergroup significant differences done using the Student–Newman–Keuls post test. A *P* value of less than 0.05 was considered significant.

3. Experimental results

3.1. Uptake of ODNs by vascular smooth muscle cells

Fluorescence microscopy of cells transfected with a fluorescein tagged ODN in chamber slides showed strong uptake of oligonucleotides, both into the cytoplasm and the nuclei of VSMCs (Fig. 1). Uptake was similar at both 24 and 48 h and was observed in more than 90% of the cells. No fluorescence was seen in cells transfected with the non-tagged AMK1.

3.2. Effect of ODNs on cell viability

Analysis of LDH release into the culture supernatant as a measure of cell viability indicated no intergroup differences between any of the ODN treated groups or untreated controls ($P = 0.12$, data not shown). In addition, no differences were noted either in cellular morphology or in the numbers of detached cells visible by phase contrast microscopy at the time of termination of the cell cultures.

3.3. Effect of sense and antisense ODNs on cell proliferation

Analysis of the tritiated thymidine data revealed that antisense ODNs produced a significant downregulation in cell proliferation (Fig. 2). There was no evidence of any toxic effect of the lipofectin alone, or of any effect of either the sense oligonucleotides or the random sequence, as there were no significant differences between any of these groups.

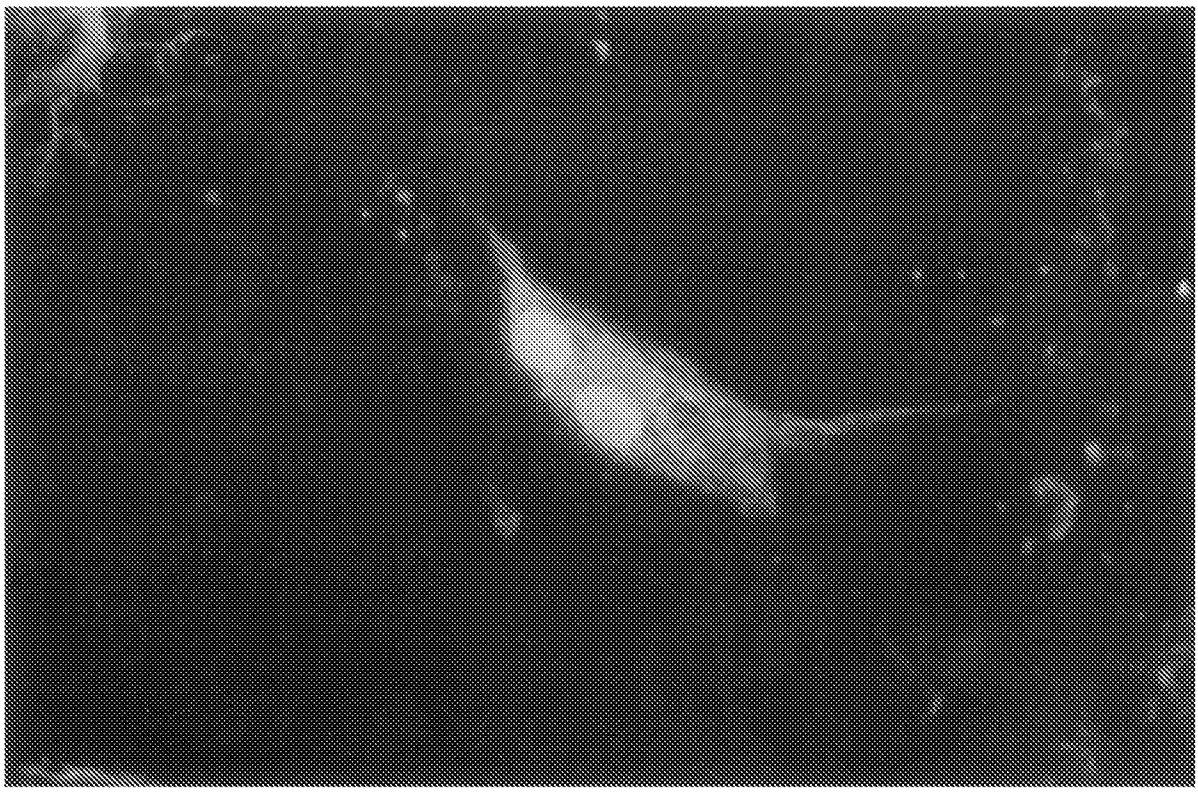

Fig. 1. Fluorescence photomicrograph of porcine vascular smooth muscle cells taken 24 h after transfection with the fluorescein linked oligonucleotide. Note the uptake into both cytoplasm and nucleus.

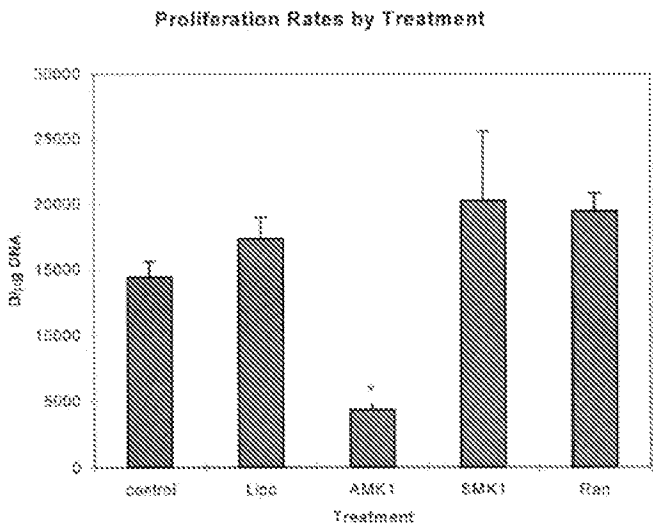

Fig. 2. Proliferation rates of cultures treated with different ODNs. Data are shown as disintegrations per minute per μg DNA. Control — untreated cells. Lipo — cells treated with lipofectin but no ODN. *$P < 0.01$ vs. the corresponding sense control and the three control groups, Control, Lipo and Ran.

3.4. Effect of antisense ODNs on ERK protein levels

The Western blotting results indicated that ERK protein levels were downregulated by the antisense ODN AMK1 compared both to its sense control (SMK1) and to untreated controls. These experiments were performed four times and in all cases the pattern of the results was the same. Fig. 3 shows both a representative Western blot derived from these experiments and also the quantitative data.

4. Discussion

Our results demonstrate that antisense oligonucleotides to ERK were successfully introduced into VSMCs by lipofectin transfection, inhibited serum stimulated growth of these cells in vitro and downregulated ERK protein expression. Previous studies performed in cardiac myocytes have demonstrated that depletion of ERK using antisense oligonucleotides inhibits the phenylephrine-induced hypertrophic response [20]. Other studies have demonstrated that the MAPK system is instrumental in transducing the epidermal growth factor induced proliferative response in vascular smooth muscle cells [17] and also is involved in the intracellular signalling responses to platelet derived growth factor and angiotensin II [24]. Binding of the appropriate ligand to the cell surface receptor leads to activation of the *ras/raf* system, which in turn activates a kinase cascade culminating in the phosphorylation of ERK itself [14]. This active form of ERK translocates to the nucleus where it leads to progression through the cell cycle through the activation of a variety of nuclear proteins [16,24].

Previous work has used antisense ODNs to target individual elements of these signal transduction pathways [21,25,26] and some of these have successfully inhibited cellular proliferation [21,25,27]. In other investigations antisense oligonucleotides have been targeted to immediate-early genes such as *c-myb* and have also inhibited cell proliferation [28]. As well as providing confirmatory evidence for the role of MAPK in cellular proliferation, the current study extends this work in two main ways. Firstly these previously described experiments have all used rodent cells, whereas we have employed porcine VSMCs. This is of relevance in that porcine cardiovascular physiology is very similar to that of humans and thus may represent a particularly relevant model for the human situation [29]. Furthermore, significant differences in response to growth stimulatory signals have been demonstrated between rodent VSMCs and those of higher mammals [30]. Secondly, the studies cited above have all used individual agonists to stimulate growth, whereas we have demonstrated that depletion of ERK can inhibit proliferation induced by the multiple factors present in serum. This model is

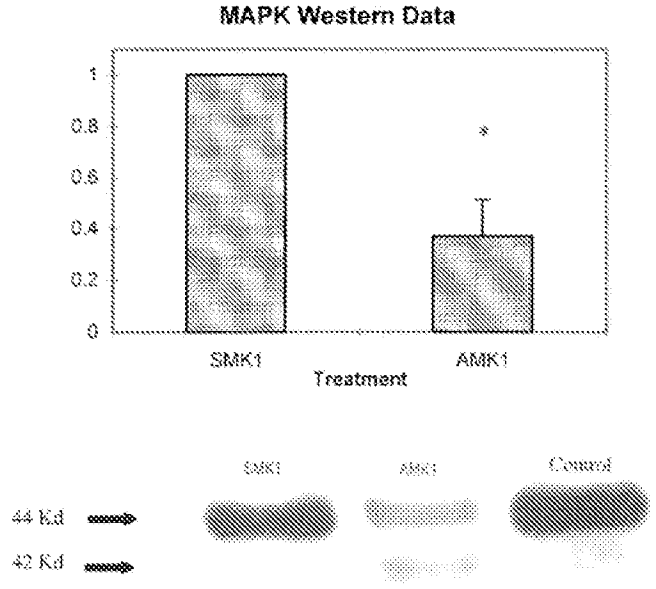

Fig. 3. (a) Western blotting data for the AMK1/SMK1 pair of nucleotides. The densitometry values were normalised in each case to produce a value for the sense oligonucleotide of one to account for exposure differences between different blots. Experiments were performed four times with the same pattern of changes noted in every experiment. *$P < 0.05$ compared to sense ODN. (b) Western blot showing the results for the AMK1/SMK1 pair demonstrating downregulation of MAPK protein by the antisense oligonucleotide.

perhaps more analogous to the in vivo situation in which cellular proliferation in response to arterial injury proceeds under the influence of a variety of regulatory molecules, as previously discussed. The work therefore supports our hypothesis that ERK represents an important final common pathway for the integration of multiple growth signals.

A potential criticism of the current study concerns the fact that porcine ERK has not yet been cloned and sequenced and thus it is not known that the AMK1 sequence is truly complementary to the 5′ end of the cDNA. In response to this it should be pointed out that the translation initiation site of both ERK-1 and ERK-2, against which AMK1 was directed, is completely conserved in all mammalian species in which the gene has been sequenced. More importantly, the object of using an antisense strategy was to downregulate the expression of ERK protein and we have demonstrated that this was achieved via the immunoblotting experiments. This reduction of ERK protein levels engendered by AMK1, associated with a reduction in stimulated cellular proliferation would be consistent with a role for ERK as a cell-cycle regulatory protein in porcine VSMCs. Nevertheless, given the non-specific effects of antisense ODNs on cellular proliferation, which have been previously noted [26], possible different mechanisms of action need to be considered. It seems highly unlikely that the effect of AMK1 was mediated via direct cellular toxicity, since LDH release into the culture supernatant was identical in all groups and there was no suggestion of differential toxicity when the cells were examined microscopically prior to termination of the cell cultures. In addition, a number of non-antisense mechanisms of action of oligonucleotides have been described which could account for these results (see Bennett [26] for a full review). Studies investigating the mechanism of action of the anti *c-myb* oligonucleotides discussed above [28], demonstrated that this effect was mediated via a non-antisense mechanism, being dependent on the presence of four successive guanine residues in the antisense ODN (the 4G effect) [31]. This is not applicable in the current study, however, as the oligonucleotides used here do not contain a 4G sequence. A further potential limitation of these experiments is the GC rich nature of the antisense sequence employed. Unmethylated GC nucleotide pairs have been demonstrated to have non-specific, inflammatory effects [32], however these do not appear to have played a significant part in these studies as both the random (RAN) and the sense control sequence (SMK1) contained an identical number of GC pairs, yet did not exhibit anti-proliferative effects. Direct interaction of oligonucleotides with cellular proteins so modifying their function has also been described [33], but this so-called aptameric effect has only been noted to occur at ODN concentrations at least 50-fold higher than those used in the current experiments.

In conclusion, we have demonstrated for the first time that antisense ODNs to ERK are capable of inhibiting serum stimulated vascular smooth muscle cell proliferation and it seems likely that this occurred via a true antisense mechanism resulting in the downregulation of ERK protein. Given the recent technological developments allowing local drug delivery to vascular angioplasty sites in humans [34,35], the study also suggests a possible role for antisense oligonucleotides to act as therapeutic agents in reducing the proliferative vascular response to injury in the clinical setting.

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